(2)

AD_____

GRANT NO: DAMD17-90-Z-0011

TITLE: IMMUNOLOGY AND PATHOLOGY OF ARENAVIRUS INFECTIONS

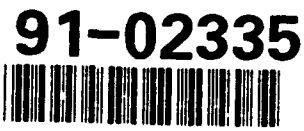
PRINCIPAL INVESTIGATOR: Norbert Herzog, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Medical Branch
Department of Pathology
Galveston, TX 77550

REPORT DATE: January 31, 1991

TYPE OF REPORT: Midterm Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012



January 31, 1991 Midterm Report (1/31/90 - 1/31/91)

IMMUNOLOGY AND PATHOLOGY OF ARENAVIRUS INFECTIONS

63002A
3M263002D807
AE
WUDA331027

Norbert Herzog, Ph.D.

University of Texas Medical Branch
Department of Pathology
Galveston, TX 77550

Grant No:
DAMD17-90-Z-0011

U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

Approved for Public Release; Distribution Unlimited

Pichinde Virus (PV) is a new world arenavirus which is the basis of a guinea pig model for human Lassa Fever. We have investigated macrophage and T lymphocyte functions to gain insight into immunity and pathogenesis in this model. Macrophages explanted from a variety of organ sources from PV infection strain 13 guinea pigs harbored infectious virus. The proportion of infected macrophages increased from 0.1% at day 6 post-inoculation to 10% by day 11. There was a significant decline in numbers of recoverable peritoneal macrophages during the course of PV infection; however, there was no overt cytopathic effect for macrophages infected in vitro over 1 96 hour period. Tumor necrosis factor (TNF)-like bioactivity was measurable in the serum of moribund animals on post-inoculation day 11, whereas TNF appeared and peaked earlier (day 6) in spleen, decreasing by day 11. Neither peritoneal macrophages explanted from infected animals nor macrophages infected in vitro constitutively produced TNF. However, in vitro, TNF production in response to LPS was augmented in PV infected macrophages compared to uninfected macrophages. These data suggest that macrophage tropism is an important aspect of PV infection and that TNFa is a complex regulated contributor to PV pathogenesis in guinea pigs.

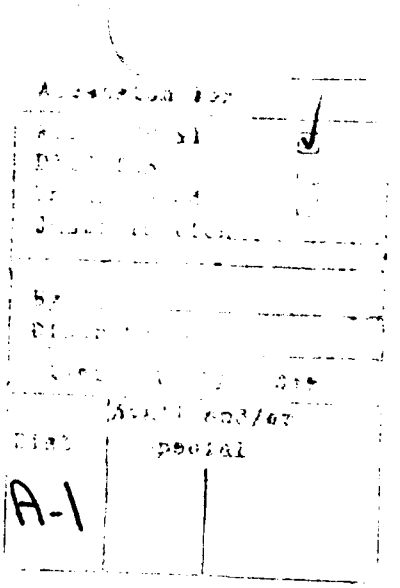
Virus, Immunology, BD, Pichinde, Guinea pigs, Arenaviruses,
RA I, Monokines

Unclassified Unclassified Unclassified Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

Block 13, Abstract (Continued)

Immunosuppression has been associated with other arenaviral infections. To explore the possibility that immune dysfunction occurs in stran 13 guinea pigs infected with Pichinde virus, we have examined the mitogenic responses of mononuclear cells isolated from the spleens and peripheral blood of infected strain 13 guinea pigs. Mononuclear cells isolated from PV-infected guinea pigs manifested markedly depressed proliferative responses to Concanavalin A (con A) and phytohemagglutinin (PHA) compared to uninfected controls. No change in the kinetics of response to mitogen was apparent in cells isolated from infected animals. Mixing experiments were performed to determine if the proliferative defect was associated with the lymphocyte or with the macrophage population. These experiments demonstrated that plastic adherence-isolated macrophages from infected animals were capable of supporting normal proliferative responses of nylon wool-isolated T lymphocytes from control animals. Conversely, nylon wool-isolated T lymphocytes from infected animals show dramatically diminished responses to mitogen in the presence of macrophages from control animals. Low amounts of infectious PV could be isolated from T lymphocytes of infected guinea pigs. These results are consistent with the conclusion that Pichinde virus infection of susceptible guinea pigs is associated with a progressive suppression of lymphocyte responses.



FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

INTRODUCTION

Human viral hemorrhagic fevers are acute, fulminating diseases, generally characterized by fever, hemorrhage, and shock. The etiologic agents include a range of geographically restricted viruses (Centers for Disease Control, 1988). Research in pathogenesis of viral hemorrhagic fevers has been hampered by the hazardous nature of these agents. The Lassa Fever virus (LFV) is a member of the family arenaviridae which also includes Junin and Machupo hemorrhagic fever viruses. Lassa Fever is endemic in West Africa, where it causes approximately 200,000 - 400,000 infections and 5,000 deaths annually (McCormick, 1987). An animal model for the West African arenaviral hemorrhagic fever, Lassa Fever, utilizes a related virus, Pichinde virus (PV), which is not pathogenic for humans. Pichinde Virus is a New World arenavirus of the Tacaribe group (Trapido, 1971). Inbred strain 13 guinea pigs are highly susceptible to PV infection, and develop a severe illness characterized by fever, wasting, and viral replication in many organs before death in the third week post-inoculation (Jahrling, 1981). The relevance of this model to human Lassa Fever stems principally from the relatedness of the viruses, and the relative paucity of histopathologic lesions in virally infected guinea pigs despite the fulminant disease course. Fatal Lassa fever accounts for 1-2% of cases of Lassa fever (Cummins, 1990), and manifests fever, facial edema, pleural effusion, adult respiratory distress syndrome, mild hemorrhagic tendencies, and "shock". Some have suggested clinical similarities to endotoxic shock (McCormick, 1986). However, pathologic findings in such fulminant cases are minimal, non-specific and do not suggest a pathogenetic sequence. Significant lesions are largely restricted to the liver in human Lassa Fever and consist of

spotty hepatocellular necrosis with minimal accompanying inflammation (Winn, 1975). Similar changes have been described in livers from PV infected guinea pigs (Lucia, 1990). Many authors have stressed that the degree of liver damage is not sufficient to account for death. This discrepancy between the severe clinical course and thc scanty histopathologic evidence of cellular damage has led to speculations that Lassa virus pathogenesis relates to either disturbance in cellular function or induction of toxic or pharmacologic mediators of fever, shock, and vascular collapse. These observations together with indications that arenaviruses are capable of infecting cells of the reticuloendothelial system (Murphy, 1977) encouraged us to investigate the role of the monokine tumor necrosis factor α (cachectin) (TNFα) in the pathogenesis of PV disease in guinea pigs. TNFα has a variety of effects including mediation of endotoxic shock, cachexia of chronic disease, antiviral properties, and others. In these studies, we have sought to delineate the degree of macrophage involvement in experimental PV infection and to document the presence of TNF activity in serum, organ homogenates and culture supernatants from macrophages explanted from infected animals.

Immunosuppression has been previously associated with PV infection and other arenaviruses. Decreased or ineffective humoral immune responses have been observed in Junin (Frigerio, 1977 and Carballal, 1981), Lassa and PV infections (Peters, 1987). Decreased mitogenic responses have been observed in Junin infections (Frigerio, 1977), and decreased ability to develop effective cytotoxic T-lymphocytes have been observed in Lassa Virus infections (Peters, 1987). Based on these observations we have explored the extent to which PV causes immunosuppression in infected strain 13 guinea pigs, and the potential mechanisms by which PV induces this dysfunction.

A well known indicator of in vivo immune function is the in vitro responsiveness of lymphocytes to mitogenic stimulation (Liener, 1986). For this reason, we began the process of characterizing T-lymphocyte function in guinea pigs infected with PV by examining in vitro splenocyte and peripheral blood mononuclear cell responses to mitogen. Subsequent to this work, we performed two studies designed to characterize the defects associated with the diminished responsiveness of lymphocytes to mitogen. The first of these consisted of mixing experiments utilizing macrophages and lymphocytes isolated from control and infected animals. We have also examined isolated T lymphocytes for the presence of infectious PV.

MATERIALS AND METHODS

Virus: PV stock was obtained from Dr. Dorian Coppenhaver, Dept. of Microbiology, UTMB as spleen homogenate from the fifteenth guinea pig passage. This strain was derived from the original guinea pig adapted strain of Jahrling et al. (1981). Viral stocks were generated in strain 13 guinea pigs as follows: strain 13 guinea pigs were inoculated with approximately 10^{3-4} PFU of the fifteenth animal passage of PV. Six days after infection, spleens from infected animals were aseptically removed, homogenized in a 10% (w:v) mixture with supplemented RPMI 1640, and then stored in aliquots at -70°C. Stocks from the 16th and 17th spleen passages were used in these experiments; stock viral titers ranged between 3 and 6 x 10^5 plaque forming units (pfu) per ml.

Concentrated viral suspensions were generated as described previously (Young, 1983). Briefly, lightly confluent monolayers of Vero cells were inoculated with PV stock dilution at an approximate multiplicity of infection of 0.04. Virus was adsorbed for 60 minutes at 37°C, then flasks were refed with Earle's modified minimal essential medium (EME) (Gibco) supplemented with 2% fetal bovine serum, (FBS), 2mM L-glutamine, 10mM Hepes buffer, and gentamicin. After 24 hours, monolayers were washed twice with Hanks Balanced Salt solution (HBSS) and refed with EME/2% FBS. Forty-eight hours post-inoculation, tissue culture supernatants were harvested and clarified by centrifugation at 1800xg for 30 minutes at 4°C. Clarified culture fluids were adjusted to 0.4M NaCl and 6% polyethylene glycol (average MW 8000, Sigma). Precipitation was carried out at 4°C for 2 hours. The suspension was centrifuged at 10,000xg for 20 minutes (4°C), the pellet was resuspended in GNTE buffer (0.2M glycine, 0.2M NaCl, 0.02M Tris-HCl pH 7.8, 0.002 M EDTA). The

material was sonicated for 37 seconds at 32 kHz in an ice bath, then centrifuged at 3000xg for 10 minutes to remove residual particulate material. Supernatants were collected and stored at -70°C. This procedure resulted in 100-fold concentration of virus over the initial tissue culture media.

Viral Plaque Assay: Virus was quantitated in a standard plaque assay on Vero cells as described previously, (Lucia, 1990), except that Vero cells were exposed to 2500 rads before plating. Briefly, irradiated Vero cells (ATCC, Rockville, MD) were incubated in supplemented EME overnight in 24 well tissue culture plates at 37°C in 5% CO_2. Diluted samples of virus were prepared in quadruplicate and were added to the wells in 0.1 ml. After incubation for one hour, at 37°C and 5% CO_2 viral inocula were removed and, 1 ml of methyl cellulose overlay media containing 50 μg/ml gentamicin, and 2% fetal bovine serum (Hyclone Laboratories, less than 0.07 ng endotoxin per ml) was added to the wells. The plates were then incubated at 37°C and 5% CO_2 for 6 days and then 1 ml of a 0.01% neutral red (Gibco) solution in overlay media was added. PV plaques were counted 8-12 hours later.

Guinea Pigs: Strain 13 guinea pigs (350-600 grams) obtained from Crest Caviary (Mariposa, CA) were free of specific detectable pathogens, and were housed in microisolator cages. For macrophage studies, guinea pigs were inoculated intraperitoneally with 2-4x10^3 pfu virus in spleen homogenate or with diluent alone on day 0. For studies of immunosuppression, all test animals were infected by intraperitoneal injection of 2 x 10^4 PFU of passage number 16 PV in 1.0 ml of RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Daily weights and rectal temperatures were recorded. Guinea pigs

were euthanatized by exsanguination by cardiac puncture under ketamine/xylazine anesthesia.

Macrophage culture: Resident peritoneal macrophages were harvested by aseptic lavage of the peritoneal cavity with ice cold, calcium magnesium-free HBSS supplemented with gentamicin. Recovered cells were washed once with HBSS and resuspended in RPMI 1640 (Biofluids) supplemented with 10% fetal bovine serum (Hyclone, less than 0.07 ng endotoxin per ml), 2mM L-glutamine, $2x10^{-5}$ M 2-mercaptoethanol, and 20 μg/ml gentamicin sulfate. Cells were counted in a Coulter counter, and differential leukocyte counts were performed on Diff-Quick (Baxter S/P) stained cytospin preparations. These suspensions usually ranged between 65% and 90% macrophages based on morphology. Cells were cultured in 24 well plates (Gibco) at a concentration of 10^6 per ml. After an 8-16 hour incubation at 37°C in a humidified 5% CO_2 atmosphere, non-adherent cells were removed by washing plates 3 times with warmed media and cultures were refed with supplemented RPMI alone or media containing 10 μg/ml E. coli O111:B4 LPS (Difco). Supernatants were harvested 24 hours later for assay of mediators. Alveolar macrophages were obtained by cannulation of the distal trachea and lavage of the lungs with ice-cold HBSS. Cells were washed once, counted and cultured as above. These populations normally consisted of >90% macrophages. For preparation of spleen macrophage cultures, single cell suspensions of spleen were made in HBSS. Red blood cells were lysed in 0.83% ammonium chloride lysing buffer and the remaining cells were washed twice in HBSS. Total and differential cell counts were performed and cells were plated at 10^6 macrophages per ml. Liver sinusoidal lining cells were obtained by modifications of previously described procedure (Rubinstein, 1987).

Guinea pigs were anesthetized and treated with 2000 U heparin by intracardiac injection. The thoracic inferior vena cava and portal veins were cannulated, and the liver was perfused retrograde or antegrade at room temperature with calcium magnesium-free HBSS with gentamicin, followed by 0.5mM ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N'N'-tetraacetic acid (EGTA) in phosphate buffered saline (PBS), PBS alone, and finally with 0.5 mg/ml collagenase A (Boehringer-Manheim) in HBSS. The liver was excised aseptically and minced in warmed (37°C) HBSS. The tissue was digested in HBSS containing 0.3% collagenase, 1% FBS, 0.004% DNase I (Sigma), and 2.5mM Hepes buffer with gentle mixing at 37°C for 30 minutes . The digested mixture was washed through nylon cloth with cold HBSS, and the suspension was centrifuged at 400 xg for 10 minutes at 4°C to pellet cells. Erythrocytes were lysed with 0.45% NaCl and cells were washed once more in HBSS. The suspension was overlaid on a discontinuous Percoll gradient (1.04 and 1.07 g/ml) and centrifuged at 400 xg for 20 minutes at 20°C. Cells at the interface between Percoll layers were harvested, washed twice in cold PBS, resuspended in complete culture media containing 10% FBS and antibiotics and plated at a concentration of 10^6 mononuclear cells per ml. Non-adherent cells were removed as described previously. By this technique, approximately 75% of resultant adherent cells were macrophages by immunofluorescence (see below). In some experiments, macrophages were cultured on sterile glass cover slips, treated as above, and fixed in acetone or 1% paraformaldehyde for immunofluorescence microscopy.

Infectious center assay: Macrophages from infected or uninfected control animals were plated in 24 well plates at varying concentrations. After washing to remove non-adherent cells and free virus, macrophages were overlaid with $2x10^5$ Vero cells previously treated with

2500 rads. The number of adherent cells was estimated visually for each separate culture. Wells were overlaid with media containing 0.5% agarose, and refed on day 6 with an agarose overlay containing 0.01% neutral red. Plaques were counted 12 hours later. Results are reported as plaques (infectious centers) per 1000 adherent cells, based on the visual estimate of adherent cells. "Plaque lifts" were achieved by transferring putative viral antigens in plaque containing monolayers to nitrocellulose discs. Immunostaining for absorbed viral protein was performed using a guinea pig anti-PV antiserum; signal was amplified and developed using a Vectastain ABC peroxidase kit (Vector Lab).

In vitro infection of macrophages: Resident peritoneal macrophages and splenic macrophages were obtained from healthy outbred Hartley guinea pigs and cultured in 24 well plates as described above. After 18 hours in culture, non-adherent cells were removed by washing, and wells were inoculated with dilutions of stock PV concentrated from tissue culture supernatants as described above.

Viral dilutions were made in tissue culture media; target multiplicities of infection were 10, 1, and 0.1. Viral inocula were allowed to adsorb at 37°C for 2 hours. Inocula were removed and macrophages were refed with complete RPMI 1640 alone or media containing 10 μg/ml E. coli LPS. To quantitate apparent cytopathic effect, adherent cells were counted at 24 hour intervals post-inoculation. Cells were counted in 3 separate medium power fields (20X) in duplicate wells using a Nikon inverted tissue culture microscope; cell counts were adjusted to numbers per mm^2 using a stage micrometer, and are reported as mean of the six counts with attendant standard deviations. Macrophage supernatants were harvested at 24 and 72 hours post-inoculation for TNF determination. Replicate wells at each time point

were overlaid with irradiated Vero cells and used in an infectious center assay to estimate efficiency of in vitro infection.

Immunofluorescence Assay: Antiserum against PV raised in Hartley guinea pigs was used in IFA to detect viral antigen in cell culture. Acetone or paraformaldehyde fixed coverslips were pre-incubated in 2% normal human serum in phosphate buffered saline (PBS) to block Fc receptors. Slides were then incubated with 1:100-1:500 dilution of guinea pig anti-PV antiserum in PBS for 30 minutes, washed extensively, and incubated in FITC goat anti-guinea pig immunoglobulin G (Kierkegaard-Perry) for 30 minutes. After washing, slides or cover slips were counterstained with Evan's blue and examined under epifluorescent illumination. Control sections were treated with normal guinea pig serum in lieu of the first step antibody. To detect macrophages, a commercial mouse monoclonal antibody specific for guinea pig tissue macrophages (MR-1, Bioproducts for Science) was used in the above procedure as the first step, with normal mouse serum as a first step control.

Tumor Necrosis Factor Assay: Macrophage supernatants or organ homogenates prepared as 1:10 (w:v) suspensions in tissue culture media were assayed for TNF as previously described (Flick,1984). Briefly, L929 cells were distributed in 96 well plates at a concentration of $4x10^4$ per well. After overnight incubation to establish monolayers, the media was removed and replaced with the desired dilution (in general, serial 2-fold increments) of sample to be assayed, or recombinant murine TNFα standard (Genzyme). Actinomycin D (Sigma) was added to each well at a final concentration of 2.5 μg/ml. Plates were placed in a 37°C incubator for 18-24 hours, then washed 3 times with warmed media, fixed in 10% formalin, and stained with crystal violet. The stain was extracted with absolute

methanol, and absorbance was determined at 540 nm on an elisa plate reader. Control wells were treated with tissue culture media and 2.5 μg/ml actinomycin D only. Each specimen and dilution was assayed in duplicate. The percent cytotoxicity for each sample was calculated as shown below:

$$100 \times [1-(\text{mean } OD_{540} \text{ sample wells}/\text{mean } OD_{540} \text{ control wells})]$$

The dilution yielding 50% cytotoxicity was derived from a plot of % cytotoxicity vs. $\log_2$ dilution of sample, and reported as units of TNF after correction for dilution factors in the assay. Where indicated, units were converted to picograms by comparison with the recombinant murine TNF standard.

Isolation of Lymphocytes: Spleen and lymph nodes were then removed using sterile techniques. Blood was collected in heparin at 50 units/ml, and then diluted in an equal volume of HBSS. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of the diluted blood through Ficoll-Hypaque (Pharmacia, Piscataway, NJ) at 450 x g for 30 minutes at room temperature. Splenic lymphocytes were obtained by gentle dissociation of spleens into single cell suspensions. All cells were washed three times in cold HBSS without calcium or magnesium supplemented with 50 μg/ml gentamicin sulfate, and then resuspended in supplemented RPMI 1640. After the final wash, cells were counted with a model ZM Coulter Counter (Coulter, Luton Beds., England) and adjusted to the appropriate cell concentration in supplemented RPMI 1640 containing 5% FBS.

Lymphocyte Proliferation Assay: Proliferation assays were performed using 2.5 x 10^5 splenocytes per well in flat-bottomed 96 well plates or 1 x 10^5 PBMC's per well in round-

bottomed 96 well plates in the presence of various concentrations of mitogen. The mitogens used were concanavalin A (Con A) (Calbiochem, San Diego, CA) and phytohemagglutinin (PHA) (Connaught Laboratories Limited, Willowdale, Ontario, Canada). Cells were cultured in a final volume of 200 μl supplemented RPMI 1640 at 37°C in a humidified 5% CO_2 atmosphere for the indicated times. Proliferative responses were established by determining the incorporation of tritiated thymidine (^{3}H-Tdr) (ICN Radiochemical, Irvine, CA). ^{3}H-Tdr (0.5 μCi per well) was added to the cultures during the last 18-20 hours of incubation. Plates were harvested (PhD cell harvester), and incorporation of ^{3}H-Tdr was determined by liquid scintillation counting.

Isolation of Macrophages and T lymphocytes: Macrophages were isolated from single cell suspensions of splenocytes by adherence to plastic in 24 well tissue culture plates. Spleen cell suspensions (2.5 x 10^6 cells/ml/well) in supplemented RPMI 1640, were allowed to incubate overnight at 37°C, 5% CO_2. Nonadherent cells were then gently removed by washing the wells three times in warm HBSS with calcium and magnesium. Plates containing adherent cells were then irradiated with 3,000 rads to prevent any remaining nonadherent cells from proliferating in response to treatment with mitogen.

T lymphocytes were isolated by adherence to nylon wool, as previously described (Mishell, 1980). Briefly, splenocytes were depleted of adherent cells as described above and adjusted to 5 x 10^7 cells/ml in warm, supplemented RPMI 1640. Suspensions of splenocytes were then incubated on a prepared nylon wool column at 37°C for 45 minutes. Non-adherent cells were collected by eluting the column at 1 drop per second, and rinsing with an appropriate amount of supplemented RPMI. Adherent column cells were collected by

using a sterile syringe to force 50 mls of cold supplemented RPMI 1640 through the column. T-lymphocyte stimulation was performed by adding 1 ml of the T-lymphocyte suspension at 2.5 x 10^6 cells/ml to individual wells containing either adherent macrophages or media only. Con A was then added to the cultures, in order to achieve a final concentration of 10 μg/ml of Con A in the cultures. Cultures were incubated for 72 hours and then proliferation was determined as described above.

Determination of Infection of Lymphocytes: To determine whether isolated T lymphocytes harbored infectious PV, splenic T lymphocytes, macrophages and column adherent cells were isolated as described above, adjusted to 10^6/ml, and then frozen at -70°C and thawed once. Quantities of PV in these supernatants were determined using the Vero cell plaque assay as described above.

Statistics: Results are reported as means with standard deviations. Where indicated, statistical significance was assessed using either the Mann-Whitney U-test or Student's two tailed t-test.

RESULTS

PV infection of macrophages from a variety of organ sources. In order to determine the degree of possible macrophage involvement in the pathogenicity of PV infection, it was necessary to demonstrate that PV does infect macrophages in vivo. Infectious center assays indicate that macrophages from a variety of organ sources (spleen, lung, liver, and peritoneum) in PV infected guinea pigs harbor infectious virus (Figure 1). During the second week of infection, when viral titers in visceral organs (liver and spleen) reach 10^6-10^8 plaque forming units per gram, the percentage of macrophages infected increased from 0.2% to almost 10%. In no case did macrophages from uninfected control animals generate plaques in Vero cell monolayers. Spurious cytotoxic effects of macrophage products on Vero cells in this assay were further ruled out by confirming the presence of viral antigen in cytolytic plaques by a transfer of plaques to nitrocellulose filters and probing those filters with antisera specific for PV antigens, as described in Materials and Methods. Examination of explanted adherent peritoneal macrophages for viral antigen by IFA yielded parallel but slightly higher figures; between 5% and 18% of macrophages showed specific fluorescence, with roughly half this number (2.5-10%) expressing viral antigens on the cell surface as judged in paraformaldehyde fixed smears (data not shown). Immunofluorescence of total and adherent peritoneal cell populations indicate that adherent macrophage populations are neither enriched for nor depleted of virally infected cells. The discrepancy between immunostaining and infectious center assays suggests that a proportion of macrophages contain viral antigen (killed virus, defective virus, partially degraded viral proteins?) without being productively infected. The relatively high rate of infection of peritoneal macrophages

may in part reflect the intraperitoneal route of infection. However, PV infection is clearly supported in other macrophage populations.

Figure 2 shows that the number of recoverable peritoneal cells declined over the course of the infection. A significant lymphocytosis (10-40%) was noted in the peritoneal exudate on day 7, but resolved by day 11. It is not clear from these experiments whether the loss of macrophages reflects cytopathic effect or alterations in the kinetics of recruitment and exodus of peritoneal macrophages.

Measurement of TNF levels after PV infection in vivo and in vitro. TNF α is produced by macrophages and can have a variety of effects, many of which are features of PV pathogenicity. Therefore, we sought to determine if levels of TNF are altered by PV infection. TNF α-like bioactivity appeared in both serum and spleen of infected animals, manifesting different temporal patterns of production in the two sites. Cytotoxic activity was present in serum only very late in the disease course (day 11 and 15) when the animals were moribund and had lost >20% of their original body weight (Figure 3). In contrast, TNF-like activity appeared in spleen as early as day 3, reaching a peak at day 6-7 after infection, and then declining by day 11 (Figure 4). When corrected for dilution factors and converted to pg TNF per gram of tissue, the levels of TNFα are 100 times higher in spleen than those maximally achieved in the serum. Heating serum or spleen homogenates for 58°C for 30 minutes neither destroyed nor unmasked cytotoxic activity. Additionally, liver homogenates showed no detectable TNF activity in this assay. Our reliance on the bioassay for TNF leaves open the possibility that we are detecting TNFß (lymphotoxin) or other undefined cytotoxic principles. We have been unable to confirm whether this activity is the

macrophage product TNFα by neutralization experiments. Commercially available polyclonal antibodies to human or murine TNFα neutralized only 50-70% of cytotoxic activity in guinea pig activated macrophage supernatants, both in solution and using solid phase immunoadsorption methods (data not shown).

In an initial attempt to elucidate a cellular source for TNF, we assayed supernatants from peritoneal macrophages explanted at various times during the infection. Results derived from two such experiments are shown in Figure 5. There is no significant difference in "constitutive" TNF production by macrophages from infected animals compared to uninfected controls. However, in the first post-inoculation week, infected macrophages elaborate less TNF in response to LPS than do uninfected control macrophages. This observation holds true even in light of some variation in adherent cell proportions and culture conditions between the two experiments.

Macrophages infected in vitro behaved somewhat differently in terms of monokine elaboration. As in the ex vivo situation, infected peritoneal or splenic macrophages did not show increased baseline TNF production compared to uninfected controls. However, in this instance, PV infection augmented TNF production in response to LPS (Figure 6), in direct opposition to the situation for macrophages explanted from infected animals. In both macrophage populations, the overproduction of TNF in infected, LPS stimulated cells was most evident at 72 hours post-inoculation, suggesting that the combination of continued viral replication and the presence of the inducer LPS are required for maximal expression of this cytokine. Based on infectious center assays and viral plaque titers from cells in this experiment, the actual proportion of cells infected at all multiplicities of infection resembles that

in vivo; i.e. between 5 and 20 adherent cells per 1000 are productively infected, with an apparent approximate 2 fold increase in this number over the 72 hours of the experiment (data not shown). These figures do not take into account any loss of cells due to viral cytopathic effect. Cytopathic effect was estimated by direct counts of adherent cells; data are shown in Figure 7. Over the course of this short experiment, infected macrophage cultures showed no excess loss of adherent cells over uninfected controls. This does not rule out cell killing by virus over a longer time course, nor does it address the issue of altered adherence properties in infected cells, although the IFA data quoted above argues against this.

Demonstration of diminished responses of splenocytes and peripheral blood mononuclear cells (PBMC's) to mitogenic stimulation. Diminished responsiveness of *lymphocytes to mitogenic stimulation* in vitro, is often indicative of in vivo generalized immunosuppression (Liener, 1986). We examined the in vitro responses of lymphocytes isolated from animals at different times after PV inoculation to T-lymphocyte mitogens. Depression of splenocyte proliferative responses to Con A was seen at days 3, 7, and 11 after inoculation (Figure 8). Responses to optimal and suboptimal Con A concentrations of splenocytes isolated from animals 3 days after PV inoculation were inhibited 10 - 50% depending on the animal or concentration of mitogen examined (Figure 8A). Statistical analyses indicated that most of these decreases at day 3 were statistically significant. By post-inoculation days 7 and 11, dramatic decreases (80-95%) in the splenocyte proliferative response to optimal and suboptimal concentrations of Con A were observed (Figures 8B and C). Splenocyte responses to PHA or supraoptimal concentrations of Con A were similarly

impaired (data not shown). Additionally, dramatic decreases in PBMC responses to Con A and PHA 7 days after infection were observed (Figure 9). Decreases in responses to Con A of PBMC's isolated from infected animals 11 days after inoculation were also observed in preliminary experiments (Figure 10). All of the decreases present 7 and 11 days after inoculation were statistically significant.

Splenocytes from infected animals do not demonstrate delayed kinetics of response to mitogenic stimulation. To rule out the possibility that the above data reflects simply a delay in the responses of splenocytes infected animals to mitogenic stimulation, spleen cells from day 11 post-inoculation animals were culture for prolonged periods (72, 96, and 120 hours) before measuring thymidine uptake. Spleen cells from infected and uninfected control animals showed similar kinetics of proliferation to this mitogen, and there was a statistically significant depression in ^{3}HTdR uptake by infected spleen cells at all time points (Figure 11).

The defect(s) in mitogenic responses of splenocytes is (are) associated with the lymphocyte and not with the macrophage. Macrophages can act as accessory cells for the induction of T-lymphocyte proliferation in response to stimulation with mitogen. It is possible that infection of animals with PV may affect the function of splenic macrophages, T-lymphocytes, or both. To discern which of these was the case, lymphocytes and macrophages were isolated from spleens of PV-infected and control animals 11 days after inoculation. Lymphocytes isolated from infected animals were examined for their ability to respond to mitogen in the presence of macrophages from control animals. Additionally, lymphocytes isolated from control animals were tested for their ability to respond to mitogen

in the presence of macrophages from infected animals. In these experiments macrophages from infected or control animals supported normal proliferative responses of lymphocytes from control animals; however, lymphocytes from control animals displayed a proliferative response to mitogen more than 100 fold greater than the response of lymphocytes from infected animals in the presence of macrophages isolated from either control or infected animals (Table 1). These data are consistent with the hypothesis that PV infection alters T-lymphocyte function, but not the ability of macrophages to support mitogen induced proliferation of T cells.

Nylon wool-nonadherent T lymphocytes from infected animals contain very low amounts of plaque forming Pichinde virus. It is possible that lytic infection of T cells by PV accounts for the apparent decrease in mitogen responsiveness of unfractionated spleen cells populations. However, in several experiments, there were no gross differences in the percentages of recovered nylon-wool non-adherent cells from spleens of infected vs. uninfected animals; in each case, 25-40% of spleen cells previously depleted of plastic-adherent cells passed through nylon wool (data not shown). Plaque assays of freeze-thawed spleen cell fractions from post-inoculation day 10 guinea pigs yielded 3000-6000 pfu of virus per 10^6 nylon wool passed cells, suggesting that a maximum proportion of 0.3-0.6% nylon-wool passed cells are productively infected. This T cell enriched population was likewise twice depleted of macrophages by plastic adherence followed by nylon wool adherence. However, even a small percentage of virally infected contaminating cell populations could easily account for the small amounts of virus detected in this assay. These preliminary results suggest that T cells are infected at a low rate compared to macrophages, and that

selective viral lysis of T cells is unlikely to account for depressed mitogen responses seen late in infection.

DISCUSSION

This disease model affords the opportunity to examine questions of viral immunity and pathogenesis at several levels: 1) How does the PV-macrophage interaction alter the profile of monokine production and cytokine interactions? 2) What is the role of TNF in the arenavirus-infected organism, i.e. is it pathogenic or protective? 3) Which cells of the immune system are infected by PV? 4) In which cell types does infection by PV alter normal immune functions? Data presented here begin to address these questions. We have shown that many elements of the reticuloendothelial system are productively infected with PV. The degree to which other cells of the immune system are involved has not yet been elucidated (see below); clearly, to the extent that macrophages are orchestrators of the immune response, their infection has profound implications for immunity and pathogenesis. Previous work has indicated that PV is not frankly lytic/cytopathic for murine macrophages (Friedlander, 1984); our observations after in vitro infection of guinea pig macrophages would support this idea. The loss of peritoneal macrophages in vivo during the course of infection may reflect either cell killing by PV, or altered kinetics of peritoneal macrophage migration. Thus, there may be a decrease in recruitment of monocytes from the peripheral blood into the peritoneal cavity, or alternatively, increased exodus of macrophages from the peritoneum. It has been demonstrated that peritoneal macrophages migrate to the spleen, among other visceral sites (Roser, 1970). This traffic may provide a mechanism for viral dissemination to other macrophage-rich organs. To date, we have no further data on alterations in other macrophage populations or precursors to determine whether PV infection affects macrophage function by simply depleting macrophage numbers.

There is ample precedent for induction of monokines by viral infection of macrophages. Productive infection of a variety of explanted macrophages and macrophage-like cell lines with Sendai virus (Aderka, 1986), Influenza A (Nain, 1990), HIV-1 (Roux-Lombard, 1989) and others has been associated with increased TNFα production. In our experience, PV by itself is not as potent an inducer of TNF as Sendai virus, which rivalled LPS in potency in human peripheral blood monocytes (Aderka, 1986), or influenza A (Nain, 1990). TNF levels in PV infected guinea pig macrophages supernatants were below the limits of detection in our bioassay. However, we have observed significant enhancement of TNF production in response to LPS in macrophages infected by PV in vitro, as previously described for other viruses (Nain, 1990). TNFα expression is regulated by both transcriptional and post-transcriptional mechanisms (Beutler, 1986). It is conceivable that PV infection enhances TNF transcription, "priming" cells for translation and secretion of the protein after an appropriate second stimulus, such as LPS. This would appear to be the situation for the synergism between IFNγ and LPS (Koerner, 1987). Virus may induce TNF by a different intracellular pathway than does LPS. Goldfield et al. found that Sendai virus infection of U937 monocyte cell line resulted in induction of TNFα by a pathway which only partially overlapped that triggered by LPS; the protein kinase inhibitor 2-aminopyrine completely blocked TNF mRNA accumulation in response to LPS, but only partially decreased viral induced TNF mRNA levels (Goldfield, 1989). Distally, at the nuclear level, signal transduction pathways may converge; in another murine macrophage cell line, sequences within the TNF gene promoter required for LPS or viral inducibility were similar (Goldfield, 1990). Experiments in progress will help clarify the level at which TNF regulation is altered in our system.

Our attempts to correlate in vitro and ex vivo viral induction of TNF have uncovered a significant discrepancy. Whereas in vitro PV infection clearly enhanced TNF production to an LPS stimulus, TNF production after LPS stimulation was depressed in macrophages explanted from infected guinea pigs in the first post-inoculation week. These differences cannot be entirely attributed to different levels of viral infection; by infectious center assays and plaque titers on infected cell lysates, the rate of macrophage infection was equivalent in the two situations. Additionally, the trend toward enhancement of TNF production was seen over a wide range of multiplicities of infection in the in vitro experiment. More likely, the decrease in TNF production by explanted macrophages may reflect the influence of glucocorticoids, prostaglandin E2, (PGE_2), or other systemic suppressive factors or cellular interactions, which would not be operative in the in vitro situation. (Preliminary data suggests that infected macrophages do not overproduce PGE_2 compared to uninfected controls). Likewise, the apparent enhancement of LPS-induced TNF production after in vitro PV infection is not likely related to such lymphokine potentiators as IFNγ. In vitro infection of macrophages by PV also differs from the in vivo situation, in that the in vitro cells become infected at the same time. The enhancement of TNF production in vitro may be a function of the conditions of the infection as well. This dichotomy points out the danger of extrapolating from in vitro observations to derive pathogenetic hypotheses.

It is tempting to attribute pathogenetic significance to the association of serum TNF activity and severe disease in this model. Many of the pathologic and pathophysiologic changes seen in these guinea pigs mimic those seen in TNF-mediated endotoxic shock (Zuckerman, 1989). The profound wasting seen in these animals is reminiscent of that

produced by chronic low level TNF administration (Tracey, 1988). TNF has been shown to induce hepatic lipogenesis (Feingold, 1987), correlating with the impressive histologic fatty change seen in moribund PV infected guinea pigs. TNFß (lymphotoxin) is a T-cell derived lymphokine which shares many biological properties with cachectin/TNFα, including the ability to kill L929 cells (Paul, 1988). Although the common pro-inflammatory properties of the two TNF's have been emphasized, only TNFα has been implicated in metabolic and shock phenomena (Paul, 1988). We have not proven that cytotoxic material is in fact the monokine TNFα. Because of the lack of availability of guinea pig specific anti-TNF reagents, we have been limited to the use of the standard bioassay for TNFα determinations. Possible spurious sources of cytotoxicity include direct viral lysis of L929 cells in the assay; we feel we have ruled this out by demonstrating that heating specimens at 60°C for 30 minutes [conditions which inactivate virus - (Allison, 1985)] did not significantly change cytotoxic activity. The activity we routinely measure in macrophage supernatants has many features of cachectin-i.e. it is heat stable, LPS-inducible, and produced by adherent cell populations with no detectable lymphocyte contamination. We have less basis for identifying cytotoxic activity in serum or spleen homogenates as cachectin, however. We are currently trying to produce neutralizing antisera to guinea pig TNFα. Additionally, Northern blot analyses are underway to determine whether the levels TNFα mRNA are altered in organs or in explanted macrophages from infected animals in comparison with uninfected animals. We will also compare these results with alterations in TNF mRNA in macrophages infected <u>in vitro</u>.

The physiologic role of circulating TNF is not clear. TNFα has a very short half life in the serum (Tracey, 1988), and is found only inconstantly in sera in a variety of human infectious and neoplastic diseases (Fong, 1990). A cell-associated form of TNF exists and may play an important in focussing local inflammatory effects of this potent monokine (Kriegler, 1988). In a rat model of burn and sepsis, cell-associated TNF in liver tissue was expressed earlier and more constantly than serum TNF (Keogh, 1990). Our finding of TNF-like activity in spleen homogenates in the first week of arenavirus infection also implies that local production of TNF has an important immunologic role. Our assay conditions of clarified spleen homogenates do not exclude the possibility that we are measuring a cell associated form of TNF. In our hands, lysates of cultured, LPS-stimulated macrophages demonstrate significant levels of TNF-like bioactivity. In vitro, TNFα demonstrates antiviral effects that synergize with but are separate from interferon properties (Mestan, 1986 and Wong, 1986). We hypothesize that the expression of soluble and/or cell-associated forms of TNF in the spleen of PV-infected guinea pigs during the period of most rapid viral replication represents a facet of the physiologic inflammatory, immune response aimed at controlling the infection. To the extent that the immune response can be separated into "protective" and "pathogenic" arms, the later appearance of TNF in the serum may represent the overflow of this mediator after the failure of local control of viral replication; circulating TNF may then trigger the production of a host of mediators associated with wasting and shock in the terminal phases of the disease. Thus, TNF and other cytokines may be viewed in this model both as mediators of the lethal effects of PV infection and as markers of an unsuccessful immune response.

We have begun to address fundamental issues of immunity in this model by documenting that PV infection is associated with profound immunosuppression in vitro. Decreased in vitro responsiveness of lymphocytes from virally infected animals often indicates in vivo immune dysfunction (Liener, 1986). Although generalized immunosuppression and secondary opportunistic infections are not features of human arenavirus disease (Cummins, 1990), immunosuppression may be of primary pathogenetic significance if arenavirus-immune system interactions impede the development of an effective specific antiviral immune response. Virus induced immune suppression is well documented in many human and veterinary diseases and experimental systems. Several mechanisms of virus-induced immune suppression have been discussed in the literature; these include direct viral infection of lymphocytes, production of host or virally derived immunosuppressive substances, increased T suppressor cell activity, or interference with various macrophage roles in the immune response (Rouse, 1986). Direct infection of T cells may cause frank cytolysis with induction of lymphopenia, as in parvovirus or HIV-1 infections; alternatively, in non-cytocidal T cell infections, differentiation and appropriate immune functions of T cells may be impaired, as in measles (Rouse, 1986). We have evidence in our PV system that hyporesponsiveness of spleen cells to T cell mitogens is not due to loss or destruction of responsive cells with nylon adherence properties of T cells. Independent confirmation that nylon wool passed cells are phenotypically T cells will be performed using monoclonal antibodies specific for guinea pig T cell markers (Elias, 1985). Based on infectious virus yields from nylon wool passaged T cell populations, we estimate that a small proportion (0.6% maximum), if any of the T cells are productively infected with PV in vivo. It would

seem unlikely that a non-cytolytic infection of such a minority of T lymphocytes could directly affect generalized T cell mitogen responsiveness.

Cultures of macrophages derived from infected guinea pigs appeared to produce more PV than did cultured nylon wool enriched spleen cells ("T cells"). Interestingly, despite the fact that macrophages are an important viral target, macrophages from infected animals were able to support mitogen-induced proliferation of normal guinea pig spleen lymphocytes. Conversely, nylon wool enriched spleen cells (T cells) from infected animals were hyporesponsive to mitogen when cultured with macrophages from infected and uninfected animals. Thus, the defect in lymphocyte proliferation of spleen cells from infected animals appears to reside in lymphocytes rather than in macrophages. The implication of a primary T cell defect leaves open the possibilities of mediation of immunosuppression by viral or host derived suppressive factors. For example, the retrovirus feline leukemia virus produces a protein P15(E) which is capable of inhibiting mitogen-induced proliferative responses of normal lymphocytes (Rojko, 1984). Interferons, particularly interferon gamma, are known to display potent immunosuppressive effects in vitro (Brenan, 1983; Rouse, 1986; Schattner, 1983). Measles virus also causes lymphocytes to produce a presently unidentified product which significantly reduces the proliferative response of T cells to mitogen (Sanchez-Lanier, 1988). Future experiments with PV will be designed to elucidate the role of soluble host or virally derived immunosuppressive factors. Additionally, other in vitro and in vivo measures of immune responsiveness, including antiviral immunity, will be assessed in this disease model. These indicators will include delayed type hypersensitivity responses, proliferative responses of lymphocytes to in vitro

stimulation with recall antigen and PV antigen, and cytotoxic lymphocyte activity against target cells infected with PV. These additional studies of immune function will further characterize the effect of PV infection on the immune systems of infected animals.

In summary, we believe that we have established a system suited for coordinated study of cytokine induced pathology and immune dysfunction. Continued investigations of PV/immune system interactions will lend valuable insights into the pathogenesis of a group of poorly understood human hemorrhagic fevers.

FIGURE LEGENDS

FIGURE 1: Productive PV infection of explanted macrophages during experimental PV infection. Macrophages from various organ sources were explanted from strain 13 guinea pigs on the indicated day after intra-peritoneal inoculation with 3000 pfu of PV. Infectious center assays were performed as described in Materials and Methods. Data represent a compilation from four separate experiments, each point is the mean of quadruplicate determinations from a single macrophage culture. AM = alveolar macrophages; PEC = peritoneal exudate cells; SM = splenic macrophages; LSLC = liver sinusoidal lining cells; n.d. = not done.

FIGURE 2: Peritoneal macrophage numbers during PV infection. Total peritoneal cell and peritoneal macrophage numbers are expressed as percentages of cells from uninfected control animals analyzed simultaneously. Data includes determinations from 5 separate experiments. In some cases, total and differential counts were not available from the same experiment, therefore the two bars of a pair may not represent the same data base. Each point represents the mean of 2 to 4 determinations.

FIGURE 3: TNF α like activity in serum of PV infected guinea pigs. Data from L929 cytotoxicity assays were normalized to pg/ml with a concurrently assayed recombinant murine TNFα standard. The lower limit of detection of TNF varied between experiments from 6 to 200 pg/ml. Values below the limit of detection in any given assay are reported as 0 pg/ml. Data is derived from three separate experiments. * Significantly different from uninfected control, $p < .05$ (Mann-Whitney U-Test). A positive control serum from a guinea pig with lethal endotoxic shock was measured at 210 ng/ml.

FIGURE 4: TNF α like activity in spleens of PV-infected guinea pigs. Spleen homogenates were prepared as 10% w:v suspensions in tissue culture media. Data is derived from three separate experiments. Results are reported as pg TNF per ml of homogenate, equivalent to pg TNF per 100 mg of spleen tissue. * Significantly different from uninfected control, $p<.05$. (Mann-Whitney U-test).

FIGURE 5: TNF production by macrophages explanted during experimental PV infection. Adherent peritoneal exudate cells from PV infected guinea pigs were cultured for 24 hours at standard concentrations in the presence (+) or absence (-) of 10 μg/ml E.coli LPS. TNF activity is expressed as pg per ml of supernatant. * Significantly different from uninfected control, $p<.05$. (Mann-Whitney U-test).

FIGURE 6: TNF production by microphages infected with PV in vitro. Resident peritoneal macrophages (A) or splenic macrophages (B) from healthy outbred guinea pigs were infected in vitro and stimulated with LPS as described in the text. Target multiplicities of infection based on projected 50% adherence of plated macrophages were 10, 1, and 0.1 for "undiluted", "1:10", and "1:100" dilutions of concentrated virus respectively. TNF activity is standardized for cell numbers based on direct cell counts at the indicated post-inoculation interval. *Significantly different from uninfected control, $p<.05$. Numbers were insufficient in (B) to apply the Mann-Whitney U-test.

FIGURE 7: Macrophage killing by PV in vitro. Resident peritoneal macrophages (A) or splenic macrophages (B) from healthy outbred guinea pigs were exposed to virus as described in the text. Direct counts of adherent cells were made at the indicated post-inoculation interval. Each data point represents the mean of six determinations from a single culture of PV infected macrophages in the absence of LPS.

Figure 8. Responses to Concanavalin A of Splenocytes Isolated from Animals on Different Days After Pichinde Virus Inoculation. Strain 13 guinea pigs were inoculated intraperitoneally with 2 x 10^4 PFU of Pichinde virus, or RPMI 1640. Splenocytes were then isolated from these animals (A) 3, (B) 7, and (C) 11 days after inoculation and were cultured with concanavalin A for 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. Each bar represents mean counts per minute of triplicate cultures from a single guinea pig. The data are expressed as mean counts per minute +/- 1 SD.

Figure 9. Responses to T-Cell Mitogens of Peripheral Blood Mononuclear Cells Isolated from Animals 7 Days After Viral Infection. Strain 13 guinea pigs were inoculated intraperitoneally with 2 x 10^4 PFU of Pichinde virus, or RPMI 1640. Blood was collected from animals by cardiac puncture, treated with heparin, and then diluted 1:2 with Hanks buffered saline solution. Peripheral blood mononuclear cells were isolated by centrifugation of the diluted blood over Ficoll-Hypaque. Isolated cells were then cultured with (A) concanavalin A or (B) phytohemagglutinin for 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. The data are expressed as mean counts per minute of triplicate cultures +/- 1 SD.

Figure 10. Responses to Concanavalin A of Peripheral Blood Mononuclear Cells Isolated from Animals 11 Days After Viral Infection. Strain 13 guinea pigs were inoculated intraperitoneally with 2 x 10^4 PFU of Pichinde virus, or RPMI 1640. Blood was collected from animals by cardiac puncture, treated with heparin, and then diluted 1:2 with Hanks buffered saline solution. Peripheral blood mononuclear cells were isolated by centrifugation of the diluted blood over Ficoll-Hypaque. Isolated cells were then cultured with concanavalin A for 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. The data are expressed as mean counts per minute +/- 1 SD of triplicate cultures.

Figure 11. Effect of Incubation Time on Responses of Splenocytes from Infected and Control Animals to Concanavalin A. Strain 13 guinea pigs were inoculated with 3 x 10^3 PFU of Pichinde virus or RPMI 1640. Splenocytes were then isolated from these animals 11 days after inoculation and were cultured with concanavalin A for (A) 120, (B) 96, or (C) 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. The data are expressed as mean counts per minute +/- 1 SD of triplicate cultures.

TABLE I

Effect of Adherent Cells from Control and Infected Guinea Pigs on Lymphocyte Responses to Concanavalin A[a]

			Source of Lymphocytes		
		Con A[b]	None	Infected	Control
Source of Macrophages	None	+	ND	5,300[c]	962
	None	-	ND	ND	604
	Infected	+	88	2,692	540,643
	Infected	-	117	404	606
	Control	+	178	3,704	442,996
	Control	-	70	372	841

[a] Lymphocytes and macrophages were isolated as described in materials and methods, from infected and control animals, 11 days after inoculation with either 2 x 10^4 PFU of Pichinde virus or RPMI 1640. Different combinations of each cell type were incubated in the presence or absence of 10 μg/ml concanavalin A for 72 hours.

[b] Designates presence or absence of 10 μg/ml concanavalin A in the culture.

[c] Proliferation was determined by ^{3}H-labeled thymidine incorporation. Values are represented as means of the counts per minute determined for quadruplicate cultures. All standard deviations were <10% of the mean, or <200 counts per minute.

LITERATURE CITED

Aderka, D., H. Holtman, L. Toker, T. Hahn, and D. Wallach. 1986. Tumor Necrosis Factor induction by Sendai virus. J. Immunol. **136(8)**:2938-2942.

Allison, L., M. Salter, G. Mann, and C.R.Howard. 1985. Thermal inactivation of Pichinde virus. J. Virol. Methods. **11**:259-264.

Beutler, B., N.Krochin, I.W. Milsark, C. Luedke, and A. Cerami. 1986. Control of cachectin (Tumor Necrosis Factor) synthesis: Mechanisms of endotoxin resistance. Science **232**:977-980.

Brenan, M., and R. M. Zingernagel. 1983. Influence of one virus infection on a second concurrent primary in vivo antiviral cytotoxic T-cell response. Infect. Immun. **41**:470-475.

Carballal, G., J. R. Oubina, S. N. Rondinone, B. Elsner, and M. J. Frigerio. 1981. Cell-mediated immunity and lymphocyte populations in experimental Argentine hemorrhagic fever (Junin virus). Infect. Immun. **34**:323-327.

Centers for Disease Control. 1988. Management of patients with suspected viral hemorrhagic fever. MMWR 1988;**37** (suppl. no. S-3):1-16.

Cummins, D. 1990. Lassa Fever. Br. J. Hosp. Med. **43**:186-191.

Elias, J. M., J. Ciba, E. M. Shevach, and H. P. Godfrey. 1985. Guinea pig T-lymphocyte development analyzed by enzyme histochemistry, monoclonal antibodies, and flow cytometry. Lab. Invest. **52**:270.

Feingold, K.R., and C. Grunfeld. 1987. Tumor Necrosis Factor Alpha stimulates hepatic lipogenesis in the rat in vivo. J. Clin. Invest. **80**:184-190.

Flick, D.A., and G.E. Gifford. 1984. Comparison of in vitro cell cytotoxic assays for Tumor Necrosis Factor. J. Immunol. Methods. **68**:167-175.

Fong, Y., and S.F. Lowry. 1990. Tumor Necrosis Factor in the pathophysiology of infection and sepsis. Clin. Immunol. Immunopathol. **55**:157-170.

Friedlander, A.M., P.B. Jahrling, P. Merrill, and S. Tobery. 1984. Inhibition of mouse peritoneal macrophage DNA synthesis by infection with the arenavirus Pichinde. Infec. Immun. **43(1)**:283-288.

Frigerio, J. 1977. Immunologic aspects of guinea pigs infected with Junin virus. Medicina (B Aires) **37**:96-100.

Goldfield, A.E., C. Doyle, and T. Maniatis. 1990. Human Tumor Necrosis Factor α gene regulation by virus and lipopolysaccharide. Proc. Natl. Acad. Sci. USA. **87**:9769-9773.

Goldfield, A.E., and T. Maniatis. 1989. Coordinate viral induction of Tumor Necrosis Factor α and Interferon ß in human B cells and monocytes. Proc. Natl. Acad. Sci. USA **86**:1490-1494.

Jahrling, P. B., R. A. Hesse, G. A. Eddy, K. M. Johnson, R. T. Callis, and E. L. Stephen. 1980. Lassa virus infection of rhesus monkeys: pathogenesis and treatment with ribavirin. J. Infect. Dis. **141**:580-589.

Jahrling, P.B., R.A. Hesse, J.B.Rhoderick, M.A. Elwell, and J.B. Moe. 1981. Pathogenesis of a Pichinde virus strain adapted to produce lethal infections in guinea pigs. Infec. Immun. **32(2)**:872-880.

Keogh, C., Y. Fong, M. Marano, S. Seniuk, W. He, A. Barber, J. Minei, D. Felsen, S. Lowry, and L. Moldawer. 1990. Identification of a novel Tumor Necrosis Factor α/Cachectn from the livers of burned and infected rats. Arch. Surg. **125**:79-85.

Koerner, T.J., D.O. Adams, and T.A. Hamilton. 1987. Regulation of Tumor Necrosis Factor (TNF) expression: Interferon Gamma enhances the accumulation of mRNA for TNF induced by lipopolysaccharide in murine peritoneal macrophages. Cell. Immunol. **109**:437-443.

Kriegler, M., C. Perez, K. DeFay, I. Albert, and S.D. Lu. 1988. A novel form of TNF/Cachectn is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell **53**:45-53.

Liener, I. E., N. Sharon, and I. J. Goldstein. (eds.) 1986. The lectins: properties, functions, and applications in biology and medicine, 1st ed. Academic Press, Inc., . (pages 355-357)

Lucia, H.L., D. Coppenhaver, R. Harrison, and S. Baron. 1990. The effect of an arenavirus infection on liver morphology and function. Am. J. Trop. Med. Hyg. **43(1)**:93-98.

McCormick, J.B., D.H. Walker, I.J. King, P.A. Webb, L.H. Elliott, S.G. Whitfield, and K.M. Johnson. 1986. Lassa virus hepatitis: A study of fatal Lassa Fever in humans. Am. J. Trop. Med. Hyg. **35(2)**:401-407.

McCormick, J. B., I. J. King, P. A. Webb, K. M. Johnson, R. O'Sullivan, E. S. Smith, S. Trippel, and T. C. Tong. 1987. A case-control study of the clinical diagnosis and of the course of Lassa fever. J. Infect. Dis. **155**(3):445-455.

Mestan, J., W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Möller, H. Jacobsen, and H. Kirchner. 1986. Antiviral effects of recombinant Tumor Necrosis Factor in vitro. Nature **323**:816-819.

Mishell, B. B., and S. M. Shiigi. (eds.) 1980. Selected methods in cellular immunology, 1st ed. WH Freeman and Co, . (pages 182-185)

Murphy, F.A., M.J. Buchmeier, and W.E. Rawls. 1977. The reticuloendothelium as the target in a virus infection: Pichinde virus pathogenesis in two strains of hamsters. Lab. Invest. **37(5)**: 502-515.

Nain, M., F. Hinder, J. Gong, A. Schmidt, A. Bender, H. Sprenger, and D. Gemsa. 1990. Tumor Necrosis Factor α production of Influenza A virus-infected macrophages and potentiating effect of lipopolysaccharides. J. Immunol. **145**:1821-1828.

Paul, N.L., and N.H. Ruddle. 1988. Lymphotoxin. Ann. Rev. Immunol. **6**:407-438.

Peters, C. J., P. B. Jahrling, C. T. Liu, R. H. Kenyon, K. T. McKee Jr, and J. G. Barrera Oro. 1987. Experimental studies of Arenaviral hemorrhagic fevers. Cur. Top. Micr. Immun. **134**:5-68.

Rojko, J. L., and R. G. Olson. 1984. The immunobiology of the feline leukemia virus. Vet. Immunol. Immunopathol. **6**:107-165.

Roser, B. 1970. The migration of macrophages in vivo. In R. van Fourth (ed.) Mononuclear Phagocytes. Blackwell Scientific Publishers, Oxford.

Rouse, B. T., and D. W. Horohov. 1986. Immunosuppression in viral infections. Rev. Inf. Dis. 8(6):850-873.

Roux-Lombard, P., C. Modoux, A. Cruchard, and J. Dayer. 1989. Purified blood monocytes from HIV 1-infected patients produce high levels of TNFα and IL-1. Clin. Immunol. Immunopathol. **50**:374-384.

Rubinstein, D., A.K. Roska, and P.E. Lipsky. 1987. Antigen presentation by liver sinusoidal lining cells after antigen exposure in vivo. J. Immunol. **138**:1377-1382.

Sanchez-Lanier, M., P. Guerin, L. C. McLaren, and A. D. Bankhurst. 1988. Measles virus-induced suppression of lymphocyte proliferation. Cell. Immunol. **116**:367-381.

Schattner, A., A. Meshorer, and D. Wallach. 1983. Involvement of interferon in virus-in duced lymphopenia. Cell. Immunol. **79**:11-25.

Tracey, K.J., S. F. Lowry, and A. Cerami. 1988. Cachectin: A hormone that triggers acute shock and chronic cachexia. J. Infec. Dis. **157(3)**:413-420.

Trapido, H., and C. SanMartín. 1971. Pichindé Virus: A new virus of the Tacaribe group from Colombia. Am. J. Trop. Med. Hyg. **20(4)**:631-641.

Winn, W.C., and D.H. Walker. 1975. The pathology of human Lassa Fever. Bull. World Health Organ. **52**:535-545.

Wong, G.H.W., and D. Goeddel. 1986. Tumor Necrosis Factor α and ß inhibit virus replication and synergize with interferons. Nature **323**:819-822.

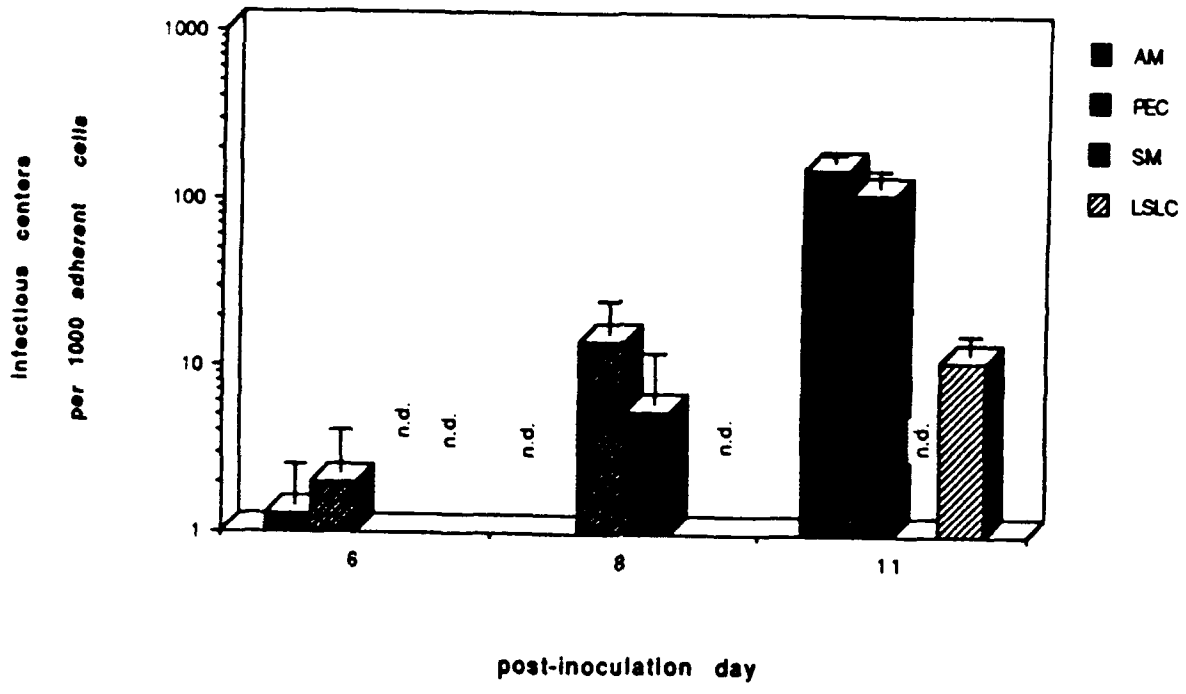
Young, P.R. and C.R. Howard. 1983. Fine structure analysis of Pichinde virus nucleocapsids. J. Gen. Virol. **64**:833-842.

Zuckerman, S.H. and A. M. Bendele. 1989. Regulation of serum Tumor Necrosis Factor in glucocorticoid-sensitive and resistant rodent endotoxin shock models. Infec. Immun. **57(10)**:3009-3013.

FIGURE 1

MACROPHAGE INFECTION BY PV

EX VIVO FOCUS FORMING ASSAY



PERITONEAL EXUDATE CELL NUMBERS DURING PV INFECTION

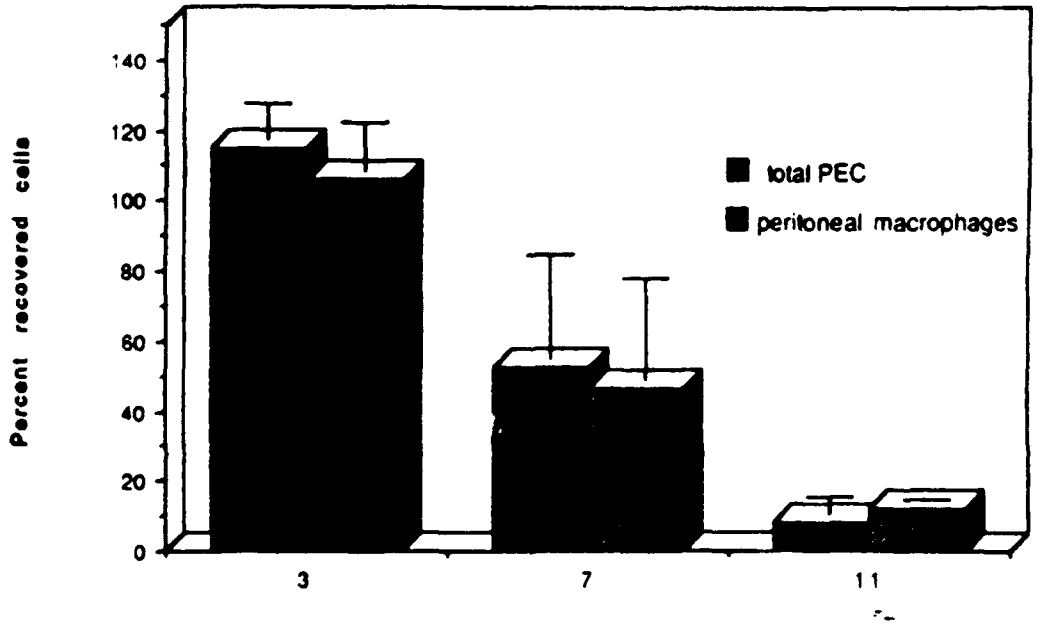
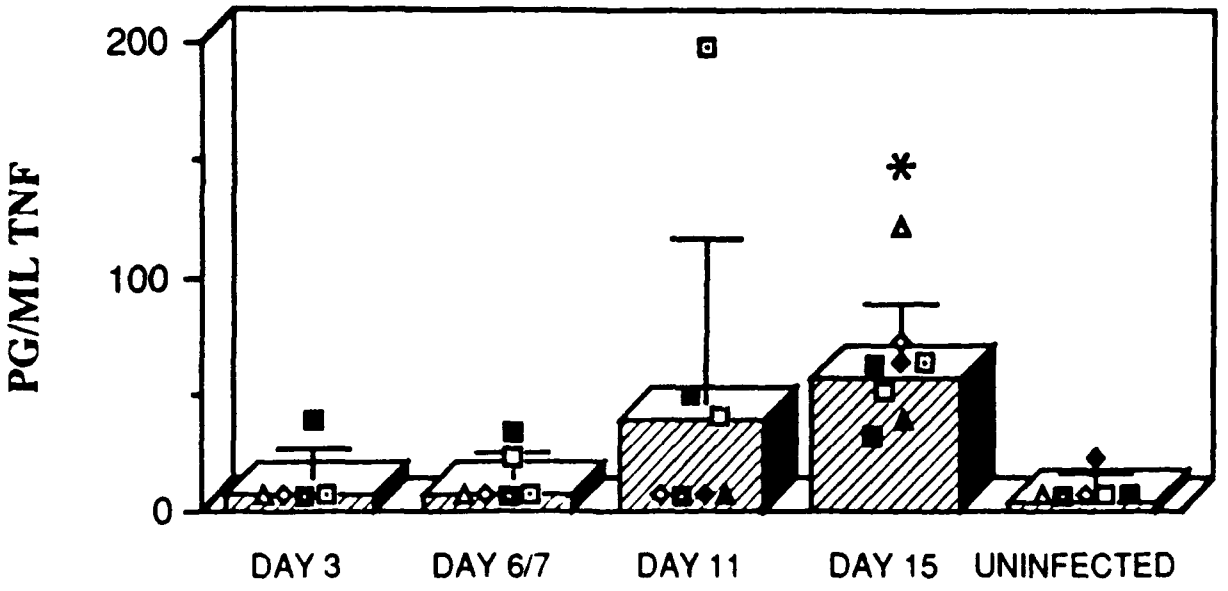


FIGURE 2

FIGURE 3

SERUM TNF LEVELS IN PV INFECTION



TNF LIKE ACTIVITY IN SPLEEN HOMOGENATES IN GUINEA PIG PV INFECTION

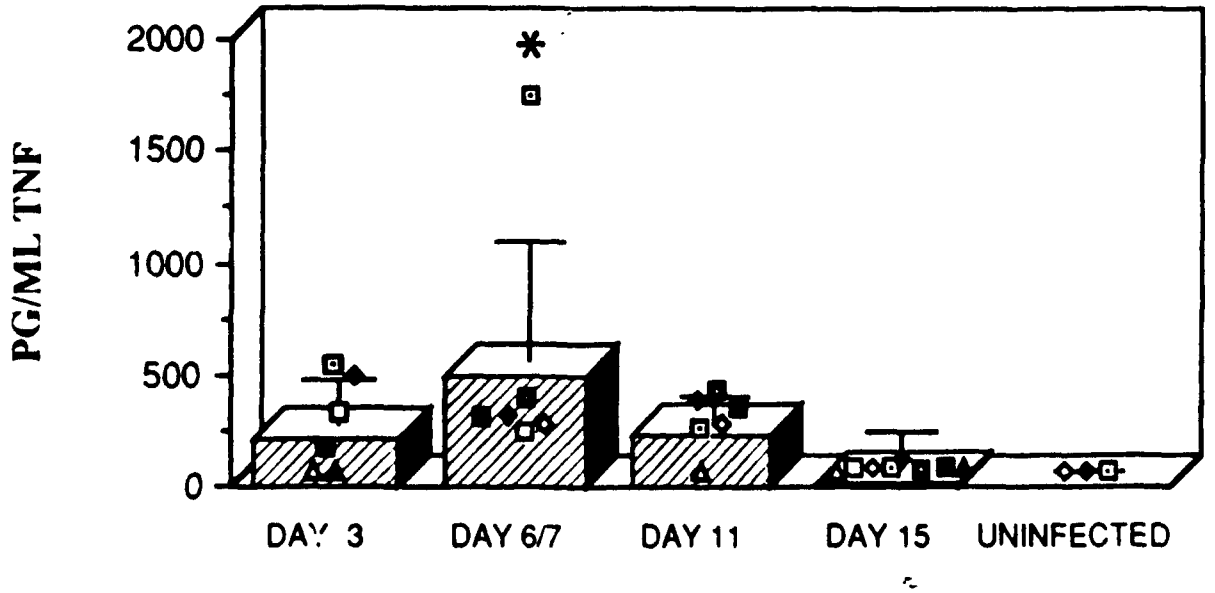
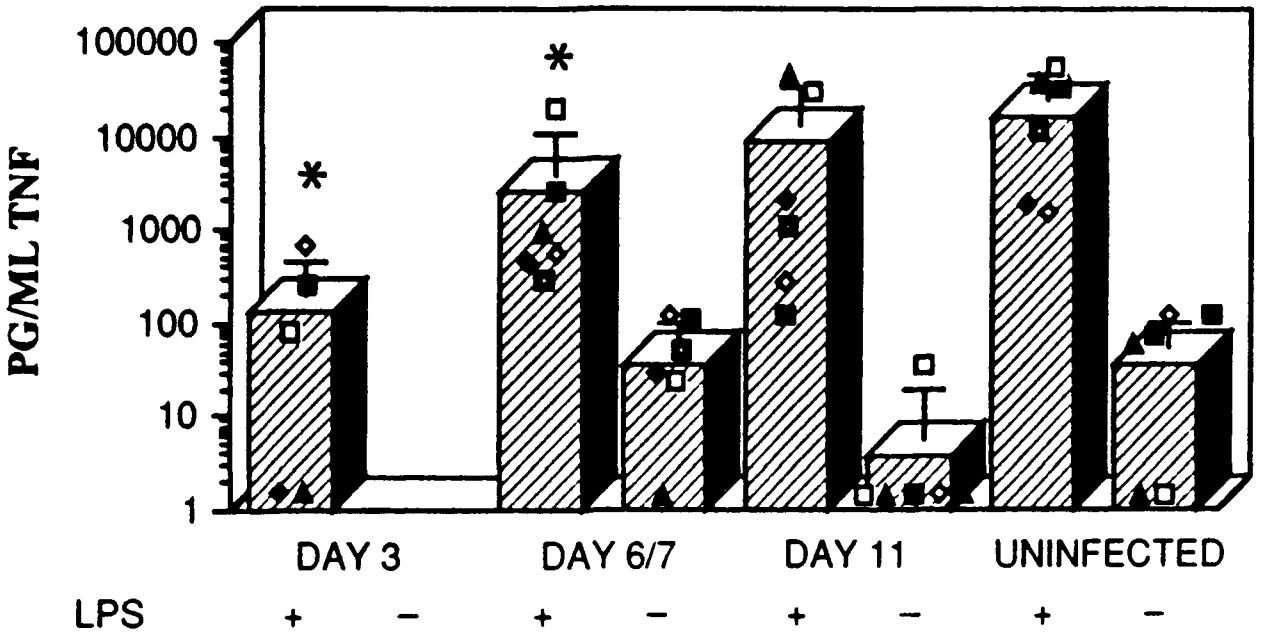


FIGURE 4

FIGURE 5

TNF PRODUCTION BY PEC DURING PV INFECTION



TNF PRODUCTION BY PV-INFECTED PEC AFTER LPS STIMULATION

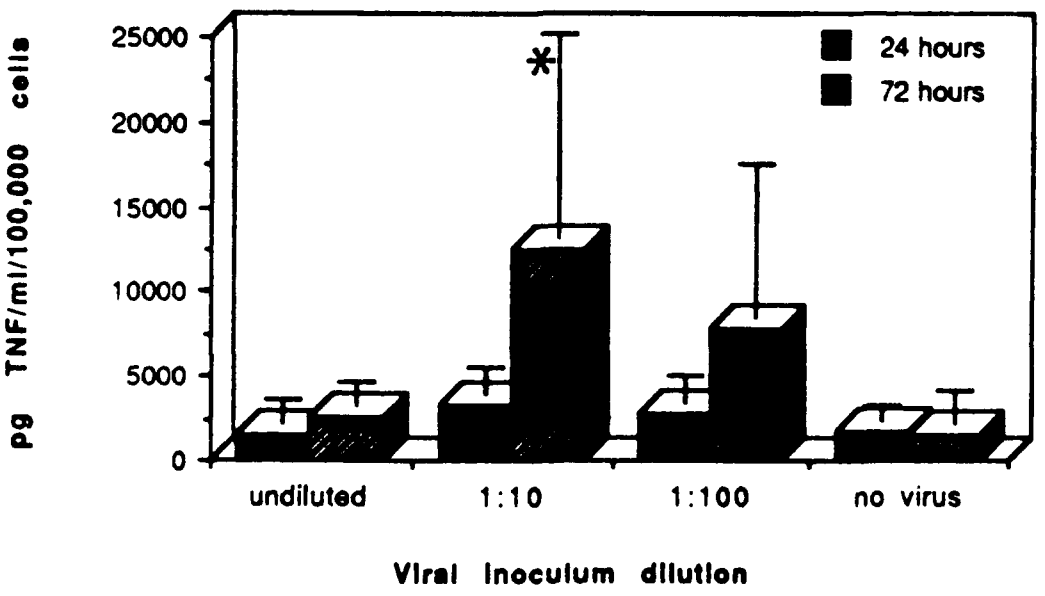


FIGURE 6a

TNF PRODUCTION BY PV-INFECTED SM AFTER LPS STIMULATION

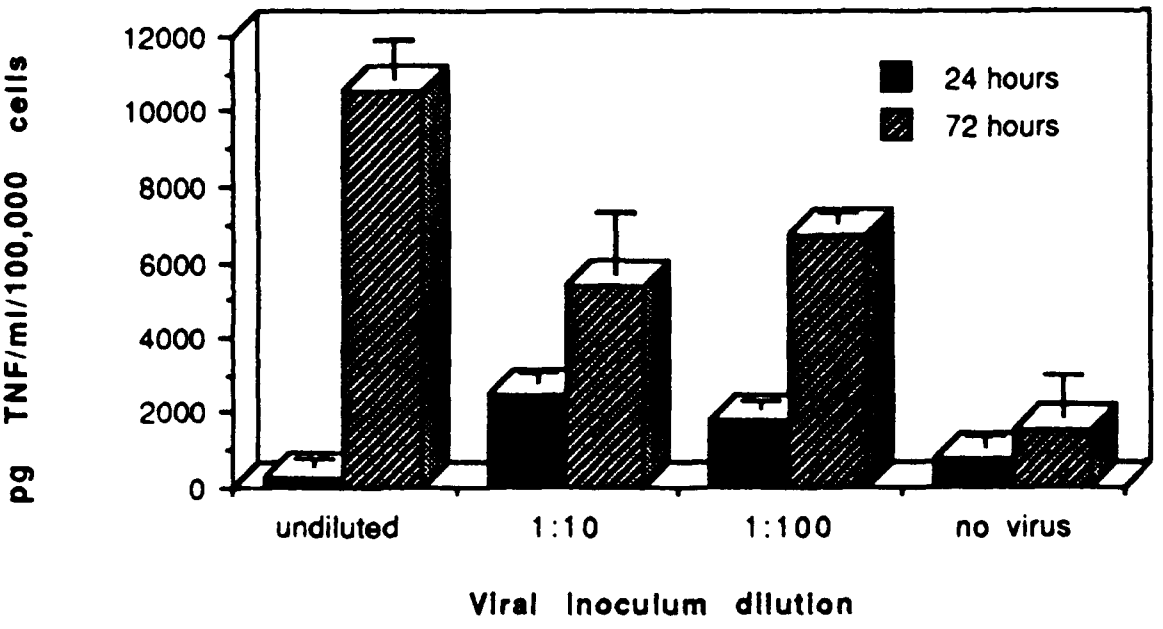
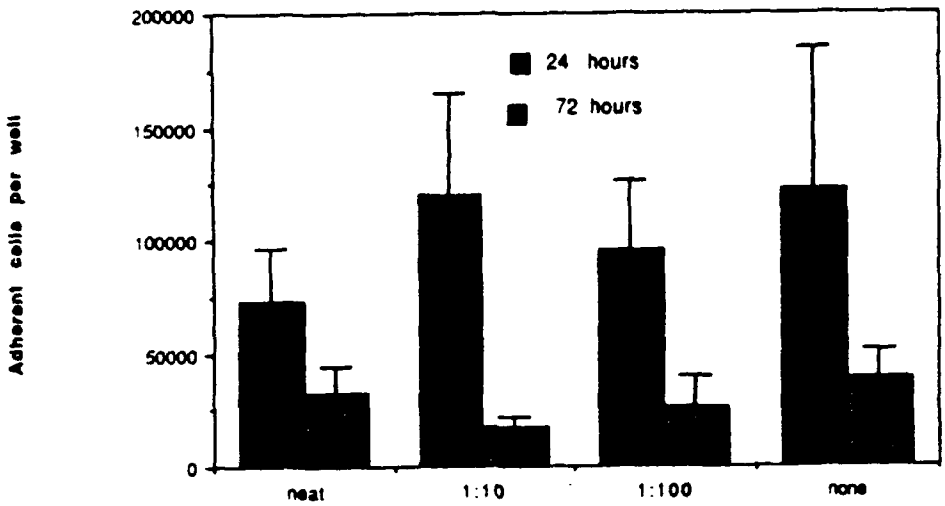


FIGURE 6b

FIGURE 7a

Cytopathic effect of PV for PEC

24 and 72 hours post-inoculation in vitro



Cytopathic effect of PV for SM

24 and 72 hrs. post infection in vitro

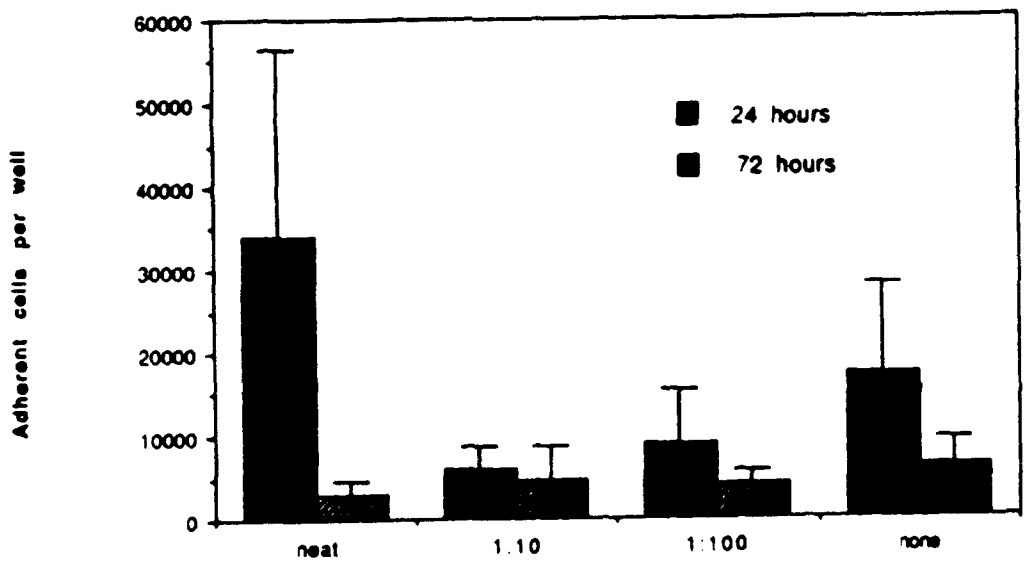


FIGURE 7b

FIGURE 8

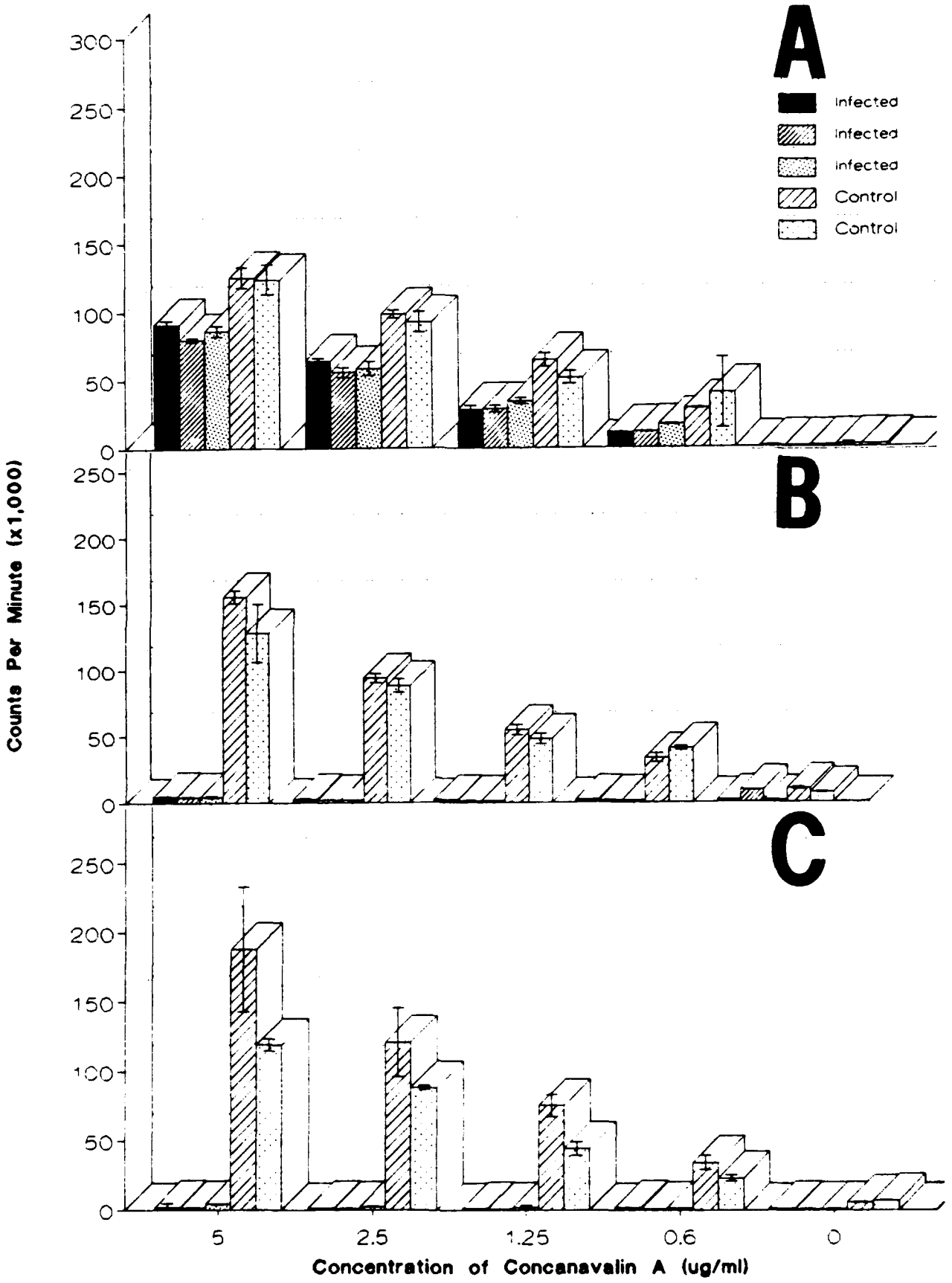

A
B
C
Infected
Infected
Infected
Control
Control
300
250
200
150
100
50
0
Counts Per Minute (x1,000)
5
2.5
1.25
0.6
0
Concentration of Concanavalin A (ug/ml)

FIGURE 9

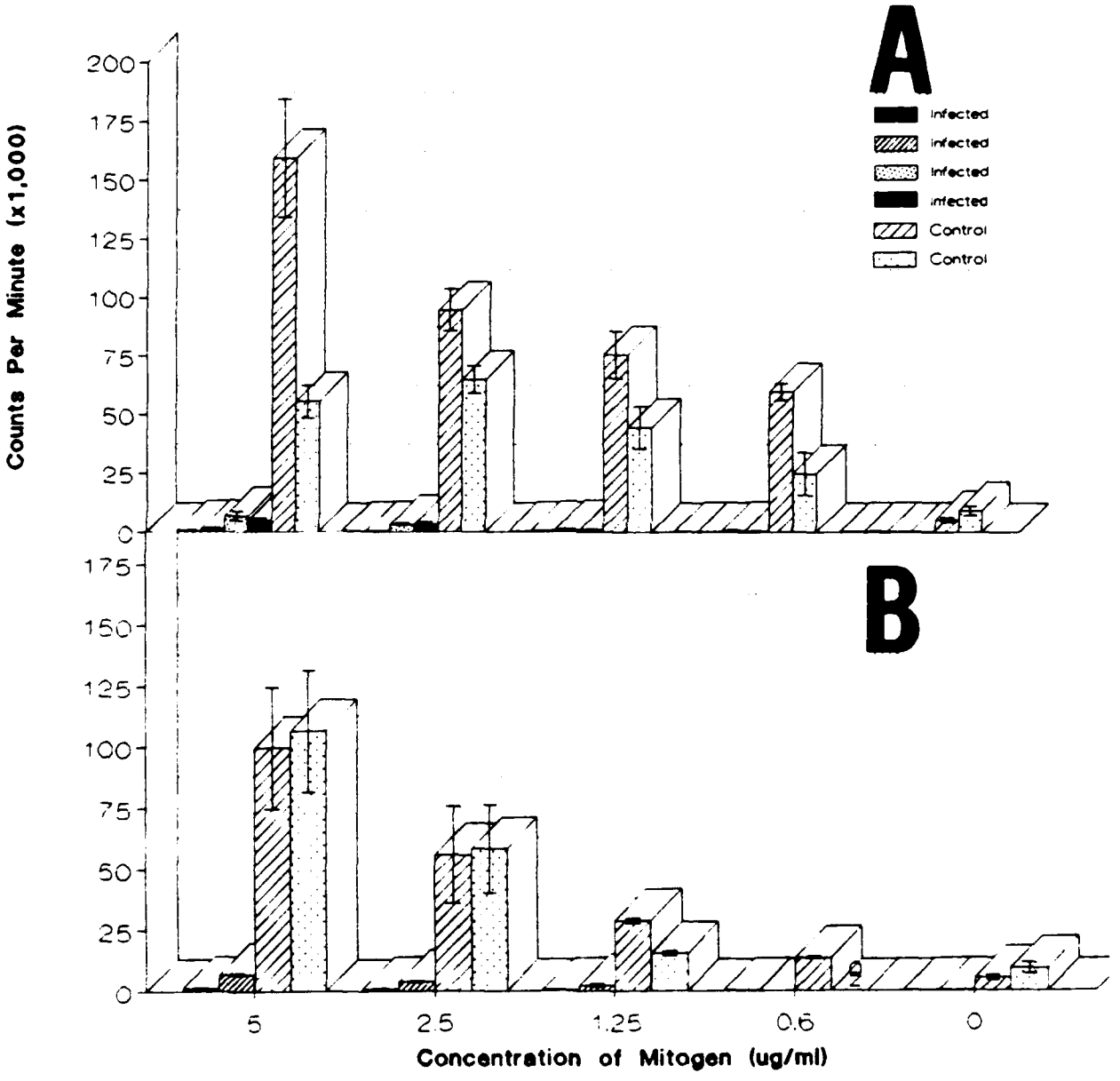

A
B
Infected
Infected
Infected
Infected
Control
Control
Counts Per Minute (x1,000)
200
175
150
125
100
75
50
25
0
5
2.5
1.25
0.6
0
Concentration of Mitogen (ug/ml)

FIGURE 10

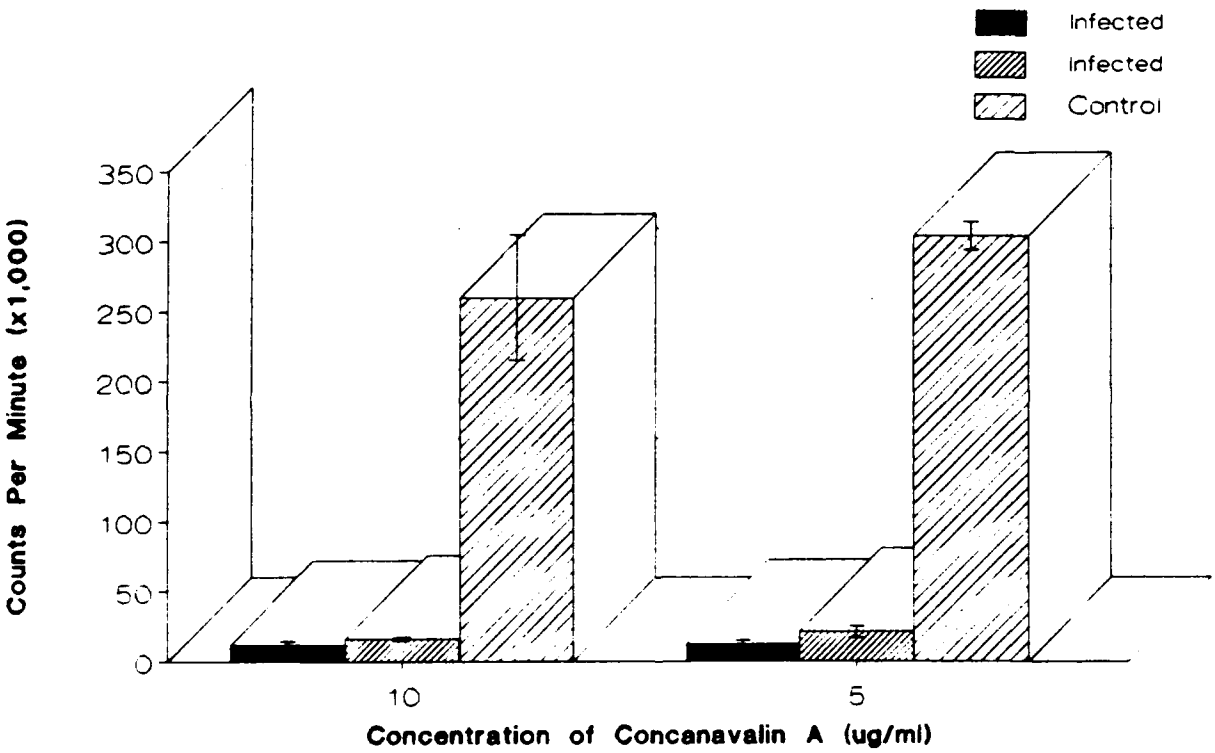
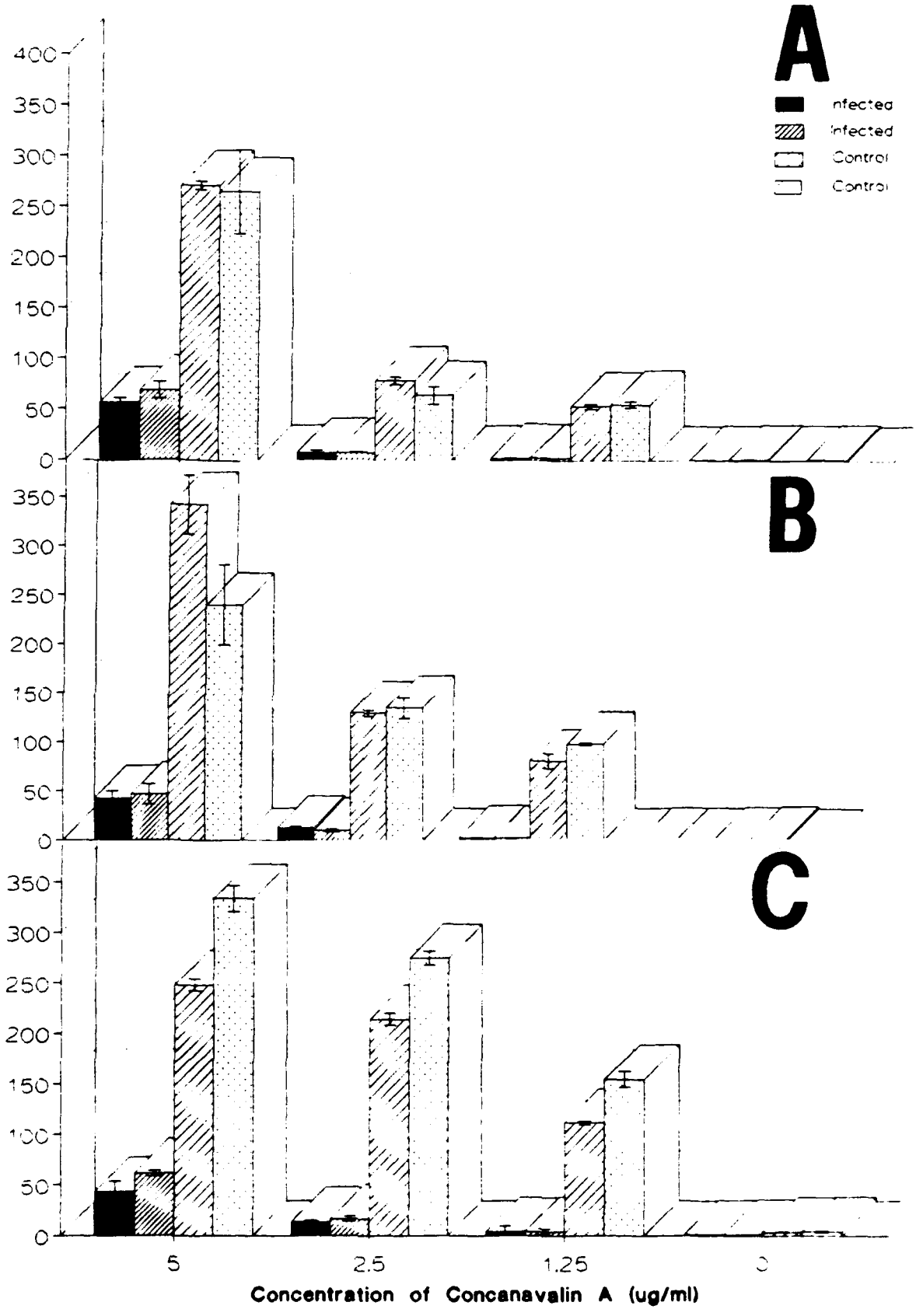

Infected
Infected
Control
350
300
250
200
150
100
50
0
Counts Per Minute (x1,000)
10
5
Concentration of Concanavalin A (ug/ml)

FIGURE 11


A
B
C
nfected
Infected
Control
Control
400
350
300
250
200
150
100
50
0
5
2.5
1.25
0
Concentration of Concanavalin A (ug/ml)